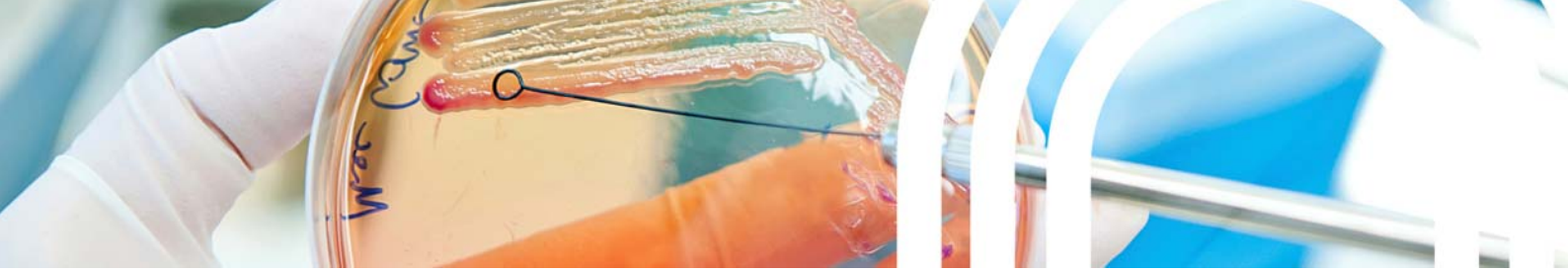

Improving together

TECHNICAL NOTE No. 6

PRECISION MODELS IN BASIC MICROBIOLOGY, *PSEUDOMONAS AERUGINOSA* AND PATHOGENIC STAPHYLOCOCCI IN CLEAN WATERS

OUTCOMES ON THE UNCERTAINTIES OF MEASUREMENTE

This document is based on the results and observations of A.G.L.A.E.'s proficiency tests

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ABSTRACT

In this technical note, A.G.L.A.E. communicates almost 15 years of experience to help you understand and interpret precision models in basic microbiology and for some pathogens in clean waters.

For the parameters considered, the evolution of the reproducibility depending on the bacterial load level is presented in order to statistically comprehend the variability of bacterial enumeration during routine analysis.

The direct links with the uncertainty of measurement calculations at the profession scale and the perspectives in terms of calculation tools are also discussed at the end of the document.

CONTENTS

1. INTRODUCTION

In parallel to the first objective of proficiency testing which is to answer the question: “are the analyses’ results of my laboratory satisfactory?”, two other questions and not the least are raised during the conduct of each proficiency test:

- What is the bacterial load level of the test materials?
- What is the dispersion (distribution of results) around this bacterial load level observed by the profession?

The statistical processing of participants’ results enables us to address these three questions for each proficiency test.
For each analytical parameter, a transversal study of data obtained for a large number of tests allows to provide an estimate of the interlaboratory dispersion at any level of the quantification range.

2. DATA

This study was carried out with data from proficiency tests conducted from 2002 in the frame of programme 30 – Microbiology in clean waters - and of programme 31 – *Pseudomonas aeruginosa* and pathogenic staphylococci in clean waters.
Depending on the parameters, the data of 23 to 42 proficiency tests were processed to produce the precision models.
The matrices used to prepare the test samples were chlorinated and neutralized drinking waters or only neutralized for some tests. For most parameters, spiking had been carried out with strains grown in broth.
The methods used by the participants for the detection and enumeration of germs mainly corresponded to French standards in use in the frame of sanitary control.

3. METHODS

3-1 Statistical processing of proficiency testing results

The means and reproducibility standard deviation were evaluated from the results of participants who had analysed during the recommended period to start the sample analysis. This enabled us not to include possible instabilities during the calculation of these statistics.
These values were calculated following the guidelines of ISO 13528, ISO 5725-1 and ISO 5725-2 standards with the use of tests detecting outliers. Except for the very low counts, the log-normal distribution is commonly used to fit bacterial enumerations counts (ISO 22117, 2010).

3-2 Development of reproducibility models

Evolution models of the reproducibility depending on the bacterial load levels were produced for each parameter. As a first step, this modelling consisted in selecting the best mathematical fitting of the reproducibility standard deviations' scatter plots s_R depending on the means m expressed in a log scale following the guidelines of the ISO 5725-2 standard.
Secondly, a chart of the squatter plots and the selected models was carried out keeping the coefficient of variation *CVR%* for the y-axis and the mean m for the x-axis, with:

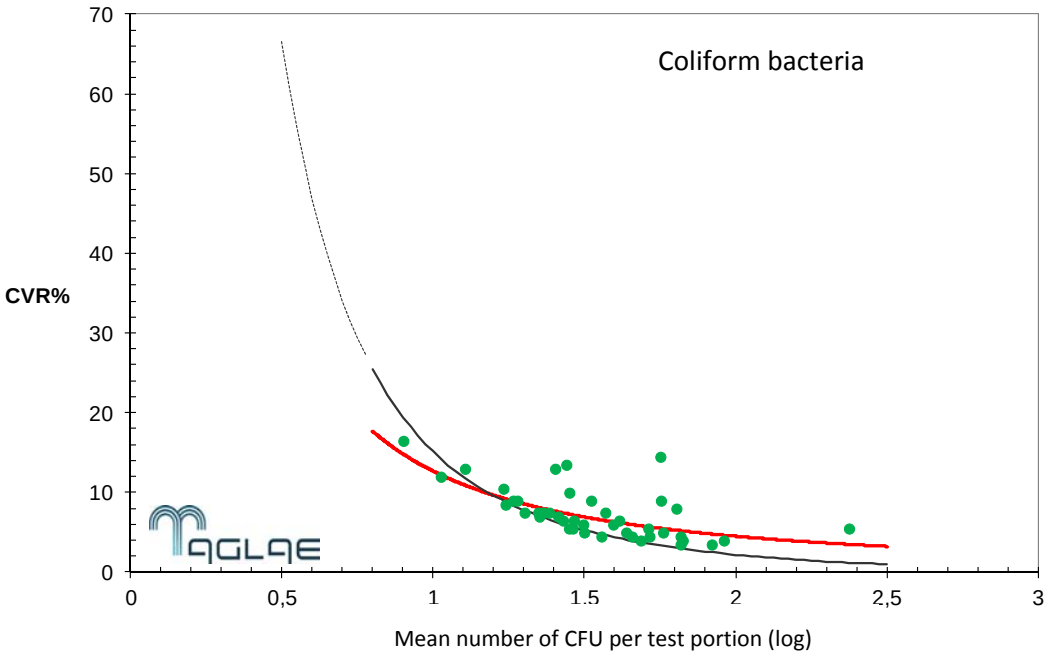
- CVR% = s_R / m × 100

On the same chart and in the same calculation scale, the curve corresponding to the intrinsic variability of any microbial count, modelled by the Poisson distribution, was also displayed.

4. RESULTS AND DISCUSSION

Results per parameter:

Figure 1: chart of the reproducibility coefficient (CVR) in % depending on the mean number of CFU in log for the parameter «coliform bacteria» (ISO 9308-1 (00))



Caption:
- Coloured points: interlaboratory comparisons conducted between 2002 and 2015
- Red curve: evolution model
- Black curve as a full line and dotted line: curve corresponding to the Poisson distribution

Figure 2: chart of the reproducibility coefficient (CVR) in % depending on the mean number of CFU in log for the parameter «Escherichia coli» (ISO 9308-1 (00))

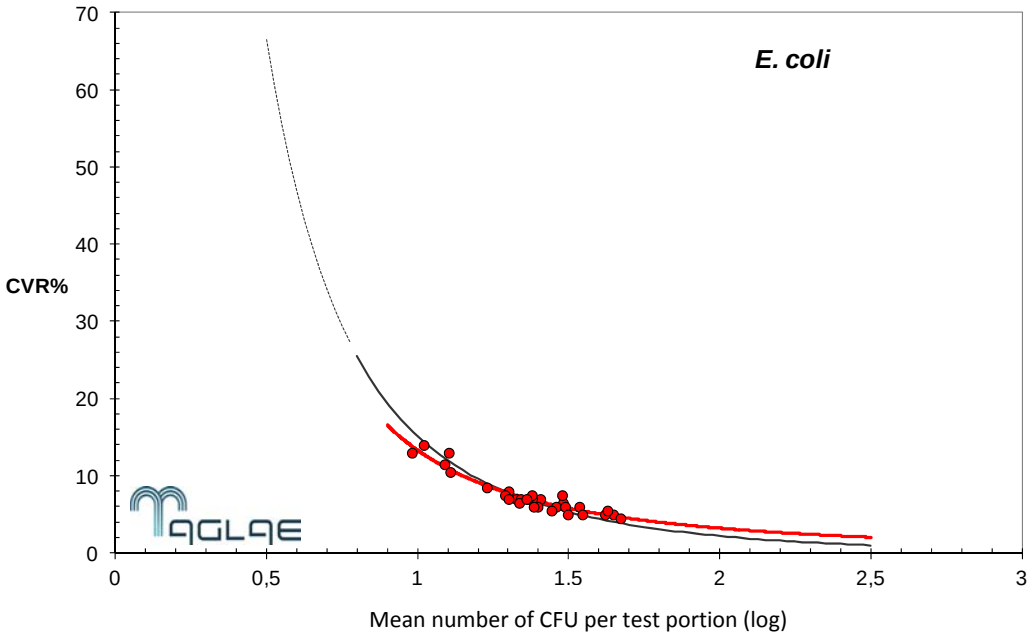
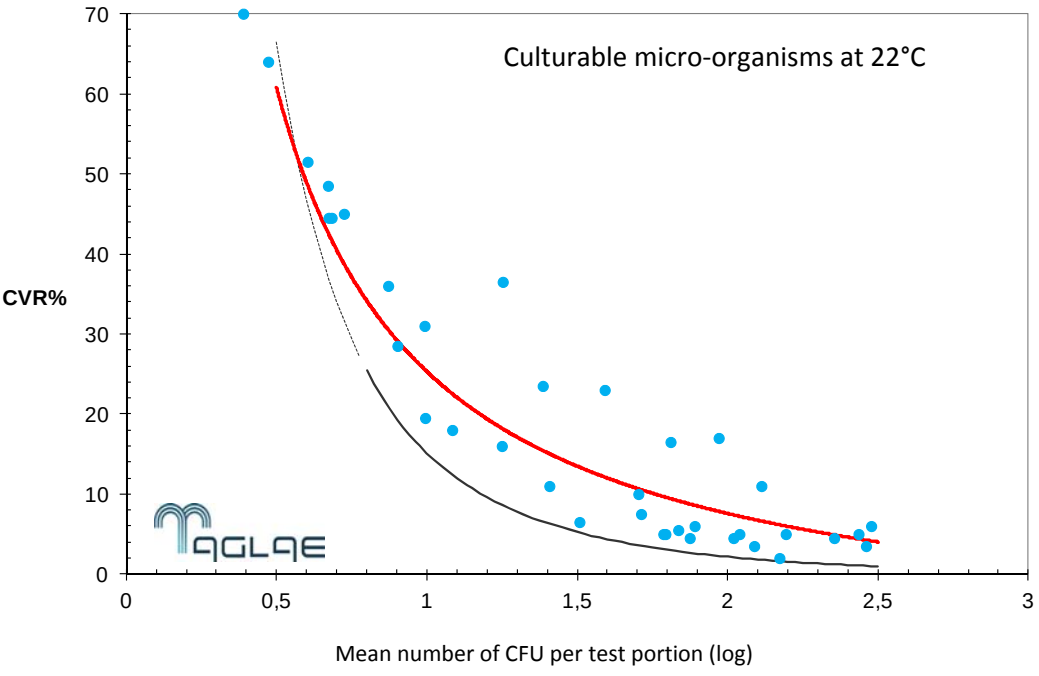


Figure 3: chart of the reproducibility coefficient (CVR) in % depending on the mean number of CFU in log for the parameter «Culturable micro-organisms at 22°C» (ISO 6222 (99))



Caption:
- Coloured points: interlaboratory comparisons conducted between 2002 and 2015
- Red curve: evolution model
- Black curve as a full line and dotted line: curve corresponding to the Poisson distribution

Figure 4: chart of the reproducibility coefficient (CVR) in % depending on the mean number of CFU in log for the parameter «Culturable micro-organisms at 36°C» (ISO 6222 (99))

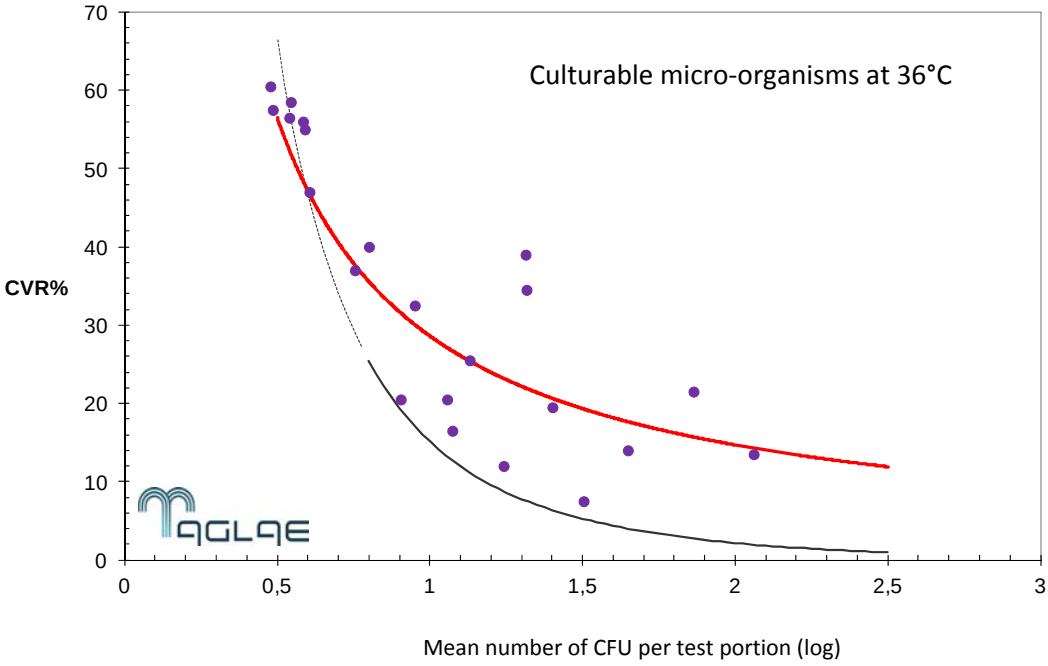
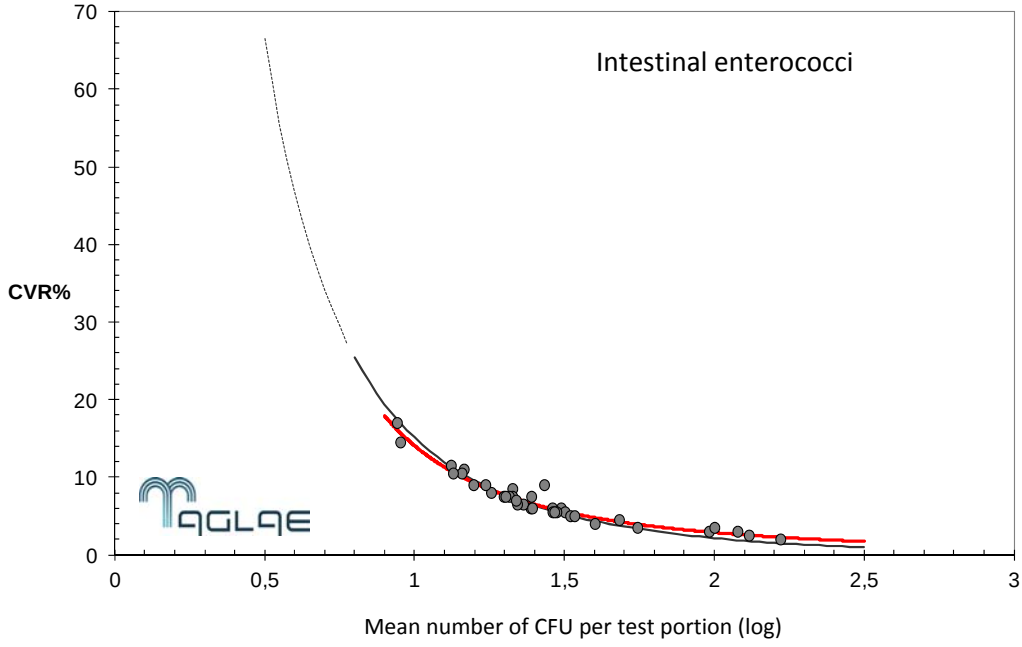


Figure 5: chart of the reproducibility coefficient (CVR) in % depending on the mean number of CFU in log for the parameter «Intestinal enterococci» (ISO 7899-2 (00))



Caption:
- Coloured points: interlaboratory comparisons conducted between 2002 and 2015
- Red curve: evolution model
- Black curve as a full line and dotted line: curve corresponding to the Poisson distribution

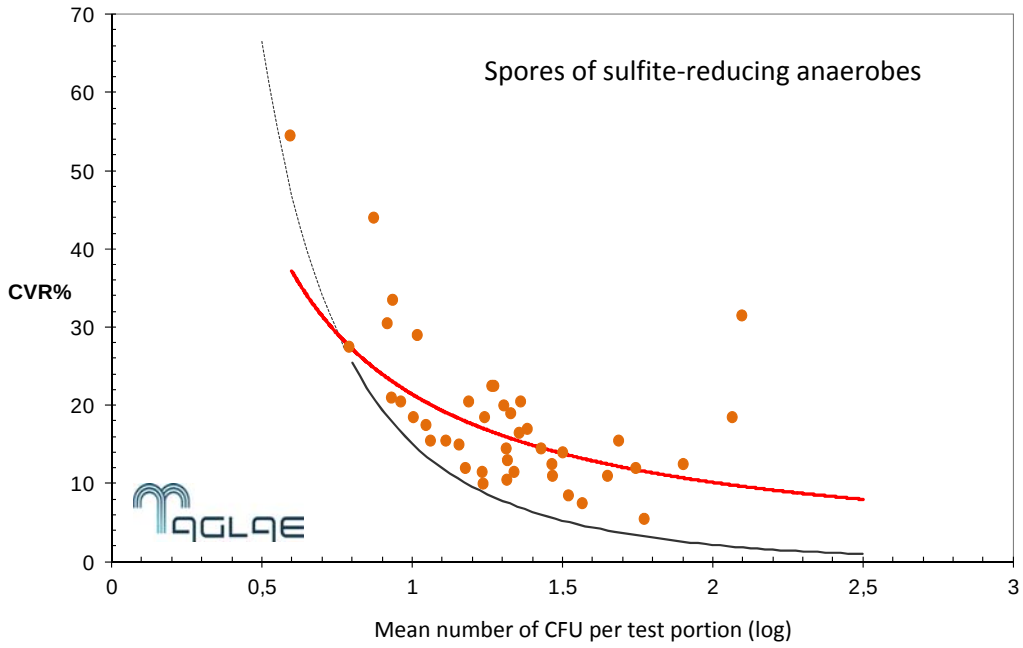


Figure 6: chart of the reproducibility coefficient (CVR) in % depending on the mean number of CFU in log for the parameter «Spores of sulfite-reducing anaerobes» (NF EN 26461-2 (93))

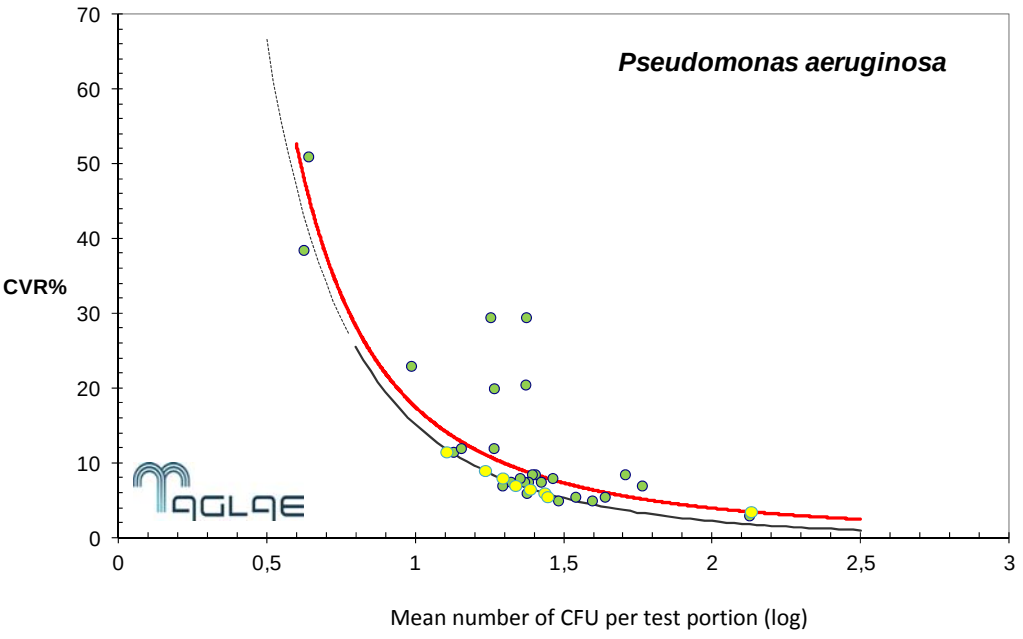
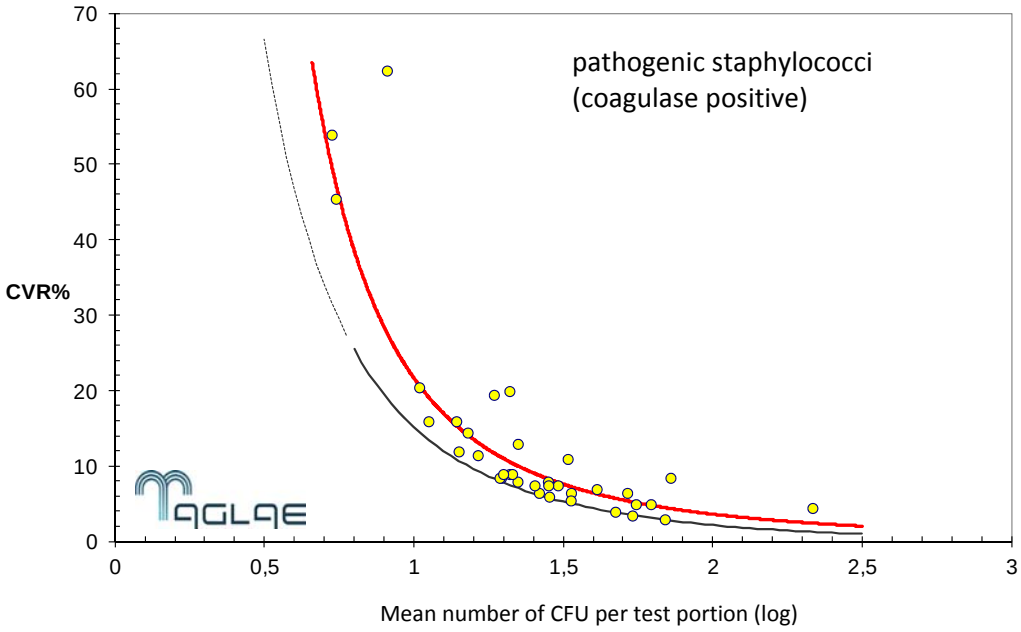


Figure 7: chart of the reproducibility coefficient (CVR) in % depending on the mean number of CFU in log for the parameter «Pseudomonas aeruginosa» (ISO 16266 (08))

Caption:
- Coloured points: interlaboratory comparisons conducted between 2002 and 2015
- Red curve: evolution model
- Black curve as a full line and dotted line: curve corresponding to the Poisson distribution

Figure 8: chart of the reproducibility coefficient (CVR) in % depending on the mean number of CFU in log for the parameter «pathogenic staphylococci (coagulase positive)» (XP T 90-412 (06))



Caption:
- Coloured points: interlaboratory comparisons conducted between 2002 and 2015
- Red curve: evolution model
- Black curve as a full line and dotted line: curve corresponding to the Poisson distribution

For each parameter, the scale illustrates **the maximal dispersion** (coefficient of variation in reproducibility) that the profession should expect **for any level of the working range**.

For example, at a mean load of 30 germs per test portion (so around 1,5 in log), the variability observed in log is:

- ✓ 6% for the enumeration of the intestinal enterococci
- ✓ 8% for the enumeration of the pathogenic staphylococci
- ✓ 14% for the enumeration of the spores of sulfite reducing anaerobes

Note: these notions of mean and in particular of variation in log can be felt as 'not intuitive' for the microbiologists of the environment. This is the reason why for the uncertainty calculations, a change of scale is usually done in order to allow a reasoning in the natural scale of enumerations (in CFU per test portion – refer to section 5).

The curve representing the **Poisson distribution** (in black) enables us to visualise the ideal case where only the random variability due to the distribution of the germs in the sample is observed during the interlaboratory tests. This curve is the one which can be kept to characterize the dispersion obtained in the most homogeneous conditions of the analysis that are the **repeatability conditions**, this for all the parameters covered in the frame of this study.

It can be noticed that for the **intestinal enterococci** and ***E. coli***, the evolution model (red curve) is very close, almost overlaid, to the Poisson distribution. This means that the results from one laboratory to another (in reproducibility conditions) are **almost totally explained by the random variability** (very close to the results in repeatability). This is the ideal case where the implementation of the standardised method in different laboratories does not produce technical disparities that can impact the results.
From a microbiological point of view, for the intestinal enterococci, the good selectivity of the Slanetz and Bartley medium coupled with the total confirmation of presumptive colonies by membrane transfer onto BEA medium can explain the good performances of the method.
The enumeration of *E. coli* has the advantage to rest upon the detection of a germ clearly defined from a taxonomic point of view. Despite of the partial confirmation by "random" picking of the presumptive colonies (oxidase and indole), the results present also a good reproducibility.

On the opposite, the **"culturable micro-organisms at 22°C and at 36°C"** are among those presenting **the highest reproducibilities**. From a technical point of view, the enumeration of the total flora according to ISO 6222 is a method that can lead to disparities due to the sensitivity of the strains or of the natural population with regard to the temperature of the poured medium and of the physiological state of the germs facing the thermal shock. It can be noted the inoculation volume of 1 mL is reduced compared to the other parameters of this study. The variability inherent in the incubation conditions (as described in the ISO 29201), could be a component contributing to a dispersion of interlaboratory results, considering the use of a non selective medium.

The enumeration of the **spores of sulfite reducing anaerobes** is the other parameter leading to **large interlaboratory disparities**. The multiplicity of analytical modalities (in particular thermal treatment, membrane porosity and anaerobiosis) and the issues regarding the robustness of the ISO 26461-2 methodology, several times highlighted in our test reports, are critical factors leading to this observation.

At an **"intermediate" level of interlaboratory dispersion**, there is the enumeration of **coliform bacteria** which, as "group of bacteria", logically presents more variability than *E. coli* on its own, as well as the two pathogens covered by the study.

For ***Pseudomonas aeruginosa***, it can be noticed that, from our observations, the type of characteristic colonies (as defined in ISO 16266) does not impact the reproducibility observed. Despite of the required acetamide confirmation step, no larger interlaboratory variability is observed when the strains introduced produce non blue-green colonies but fluorescent ones (yellow data points on Figure 7) In comparison with tests for which the colonies produce a blue-green pigmentation (green data points on Figure 7). For each of the two types of characteristic colonies, non target strains had sometimes been introduced for some tests (*Ps. putida*, *Ps. mendocina*, *Serratia rubidaea*…).

For the **pathogenic staphylococci (coagulase positive)**, it can be noted that the tests where major differences between the types of culture medium (Chapman – BP – BP+RPF) had been detected are not presented among the data points displayed, which tends to idealise the evolution model obtained. Nevertheless, the revision of the standardisation of the method XP T 90 412 to come with the use of only one type of culture medium (BP+RPF) should theoretically lead to the observation of an improved reproducibility.

This hypothesis rests, between others, on a study related to the results' variability according to the type of culture medium observed and on an examination of the effects «culture medium manufacturer» and «membranes manufacturer» within each type of medium. In order to determine a **possible effect due to the strain**, the last 32 AGLAE's interlaboratory tests for which test samples had been spiked with one strain only, were transversally processed.
A summary of the differences between types of culture medium is presented below in figure 9 for each of the 8 strains tested for these tests.

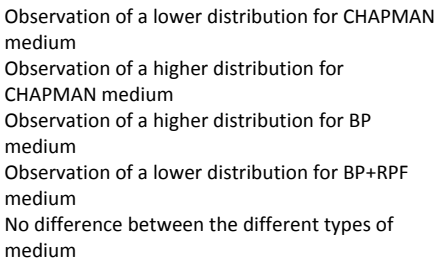


Figure 9: pie charts of significant differences between types of culture medium for the parameter «pathogenic staphylococci (coagulase positive)»

It is important to keep in mind that among the 8 strains tested, 6 of them showed significant differences detected on CHAPMAN medium (in comparison with BP and BP+RPF) whereas 2 of them showed differences on BP + RPF medium (in comparison with Chapman and BP).

On the other hand, significant differences, of greater or lesser extent, between «culture medium manufacturer» and «membranes manufacturer» were also detected within CHAPMAN and BP + RPF medium.
Table 1 here-after illustrates this information.

Strains / Observations	strain 1	strain 2	strain 3	strain 4	strain 5	strain 6	strain 7	strain 8
Differences between «medium manufacturer» CHAPMAN	**80%**	25%	25%	0%	0%	0%	**100%**	**67%**
Differences between «medium manufacturer » BP+RPF	40%	**50%**	25%	33%	0%	0%	0%	0%
Differences between «membrane manufacturer» associated with CHAPMAN medium	**80%**	**75%**	**50%**	**100%**	0%	33%	**50%**	0%
Differences between «membrane manufacturer» associated with BP+RPF medium	0%	25%	25%	0%	0%	0%	0%	0%

Table 1: Review of the number of tests where a difference was detected (in %) depending on the strain used and the type of culture medium used for the parameter «pathogenic staphylococci (coagulase positive)»

Note: for a given test, it is possible that a difference is detected for several types of medium

Through proficiency testing, BP+RPF medium seems to produce less differences between consumables' manufacturers, in comparison with CHAPMAN medium; which should theoretically reduce the disparities from one laboratory to another one if the standardisation selects exclusively the first medium.

5. OUTCOMES ON THE UNCERTAINTIES OF MEASUREMENT OF THE PROFESSION

The ultimate objective which is generally aimed during a transversal study of interlaboratory comparisons is the connection between the precision observed and the determinations of the uncertainties of measurement.

As a reminder, the uncertainty of microbiological methods consists of two components (ISO 29201, 2012):

- the **intrinsic variability** (distribution uncertainty) associated with the random distribution of germs in the sample. From a statistical point of view, it is modelled by the Poisson distribution,
- the **operational variability** (technical uncertainty) which is the combination of all the uncertainties associated with the technical steps of the analytical protocol. This is the component to evaluate.

For each chart presented in section 4, the difference between the black curve (Poisson distribution) and the red curve (evolution model) represents the operational variability (technical uncertainty) observed by the profession and expressed in a log scale.

Yet, the metric used to describe the uncertainty of measurement in microbiology is the relative variance, u^2, calculated from the number of counted colonies. From a mathematical point of view, the parameter u^2 comes from a particular expression of the variance according to the Negative Binomial distribution and represents the over-dispersion in relation to the Poisson distribution (ISO 13843, 2000). From a biostatistical point of view, this over-dispersion corresponds to the operational variability then qualified as "relative" that we are interested in evaluating.
The use of this metric allows to reason in the natural scale of the number of bacteria per test portion.

In concrete terms, to transform the variances in log to the relative variances in u^2, a conversion can be applied following the ISO 29201 guidelines:

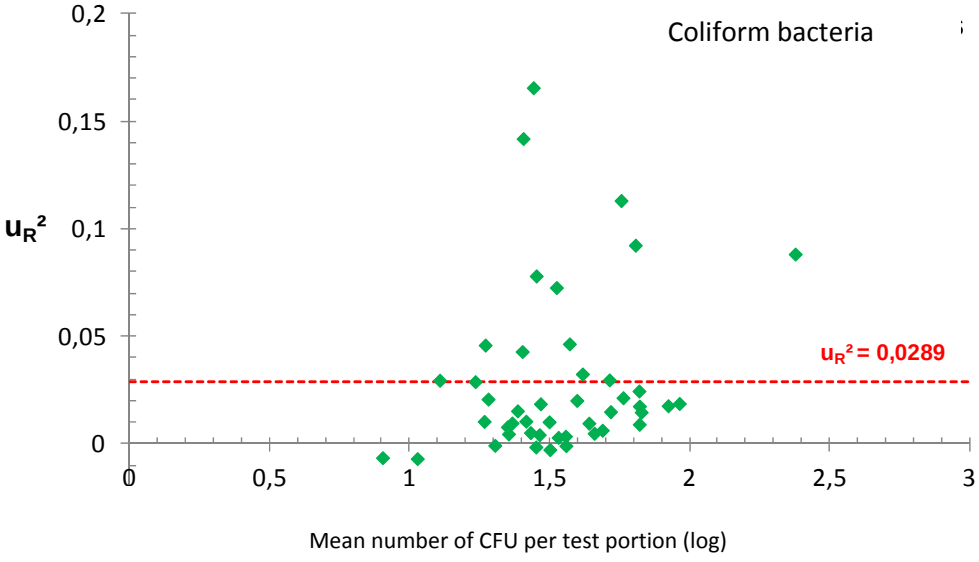
- $u_{tR}^2 = 5{,}3019\ s_R^2$ with u_{tR}^2 = relative standard uncertainty of interlaboratory reproducibility
  s_R^2 = relative standard uncertainty of interlaboratory reproducibility, expressed in log

An ultimate calculation step allows to deduce the relative operational variance and designated as u_R^2:

- $u_R^2 = u_{tR}^2 - u_{Poisson}^2$ with u_R^2 = relative operational variance of interlaboratory reproducibility (technical uncertainty in reproducibility)
  $u_{Poisson}^2$ = intrinsic relative variance (uncertainty of Poisson distribution)

Thus, for each parameter considered and for each test, the application of these conversions allows to transform the interlaboratory variability measured in uncertainty of measurement for the profession.
For example, for the enumeration of coliform bacteria, parameter for which we could highlight the extent of the reproducibility in the previous chapter, the expression of the interlaboratory variability in u_R^2 is visualised on Figure 10 below.

Figure 10: chart of the squared standard deviation of the profession, u_R^2, depending on the mean obtained for each test for the parameter "coliform bacteria"



Caption:
- Green data points: u_R^2 for the different proficiency tests conducted
- Red dotted line : mean value of u_R^2

This type of information related to the uncertainty of measurement of the profession is provided in our test reports for the programmes 30 (basic microbiology) and 31 (Pseudomonas aeruginosa and pathogenic staphylococci).
For a global estimate of the uncertainty, the operational variance is specified depending on the conditions in which the measurements were obtained. It is the general information about the profession from the FD T 90-465-1:

- u_r^2 for the repeatability conditions
- u_R^2 for the interlaboratory reproducibility conditions

Table 2 below summarises all the estimates updated for the parameters studied in this technical note.

Parameters	u_r^2: repeatability conditions	u_R^2: interlaboratory reproducibility conditions
Coliform bacteria	0,0000	0,0289
Escherichia coli	0,0000	0,0068
Culturable micro-organisms at 22°C	0,0000	0,1542
Culturable micro-organisms at 36°C	0,0000	0,1649
Intestinal enterococci	0,0000	0,0064
Spores of sulfite reducing anaerobes	0,0000	0,1725
Pseudomonas aeruginosa	0,0000	0,1041
Pathogenic staphylococci	0,0000	0,0541

Table 2: Summary of the uncertainty estimates for the profession (values available in October 2015)

Note that the u_R^2 value for each parameter is the mean of available estimates for all the proficiency tests already carried out (ex : u_R^2 = 0,0289 in figure 10 and of table 2 above).
Regarding the mean value of u_r^2, it is not significantly different from 0,0000 for a large number of proficiency tests. This is the reason why the null value was kept.

These values of u^2 can be transformed in concrete information about the uncertainty, as recommended by the document FD T90-465-1: limit of detection (LD), confidence interval (or of credibility) confidence interval around a result observed…
For example: for the enumeration of coliform bacteria in 100 mL, with u_R^2 = 0,0289

- LD = 3,13 germs in 100 mL
- For 6 confirmed colonies observed on a Petri dish:
  - result = 6 coliform bacteria in 100 mL
  - Confidence interval: between 2,16 and 13,68 coliform bacteria in 100 mL, at the α risk of 5%

The conversion modalities of u^2 in explicit information about the uncertainty are described in the document FD T90-465-2.

For information only, an empirical scale of the technical uncertainties that can be observed during routine tests is presented in figure 11 here-after in.
At the scale of the profession or at any laboratory's level, this scale can enable us to apprehend and to put into perspective the extent of any uncertainty evaluated in terms of u^2, with regard to the experience acquired in the water microbiology field.

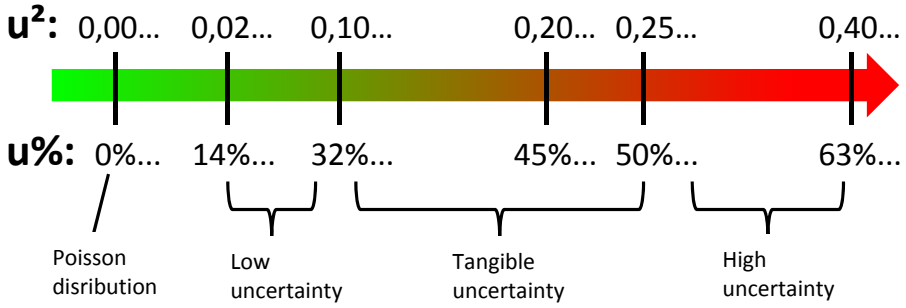


Figure 1: empirical scale of the uncertainty of measurement in water microbiology

Note : Poisson distribution: intrinsic variability (entirely random distribution uncertainty of the particles during the sampling of a perfectly homogenised suspension)
"Technical" uncertainty: operational variability corresponding to the over-dispersion with regard to the Poisson distribution, in terms of relative variance (u^2) or relative standard deviation (u% = squared root (u^2)×100)

6. PERSPECTIVES

Perspectives can be seen in terms of modelling of the microbiological measurements' variability, so that the models routinely used to process proficiency testing results rest directly on random variables known as the most appropriate from a biostatistical point of view (Poisson distribution, Negative Binomial or Gamma-Poisson).
These improvements could in particular allow to avoid the transformation of counting data which could disturb the dispersion estimates and would provide the opportunity of a total connection between the statistical indicators used for the uncertainty of measurements calculations and the ones used for the analytical performance assessment in the frame of the External Quality Control.

7. DESIGN AND ET DEVELOPMENT OF THE TECHNICAL NOTE

The present technical note was carried out by the Biology data processing team, in collaboration with the technical team and AGLAE's management and published after consulting the Microbiology Technical Committee.
We sincerely thank the members of the Technical Committee for their acute reading.

8. BIBLIOGRAPHY

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FD T90-465-2: finalisation on-going - Water Quality - Protocol for the estimation of the uncertainty of measurement associated with an analysis result for microbiological enumeration methods - Part 2: case of enumeration methods

ISO 13528:2015 - Statistical methods for use in proficiency testing by interlaboratory comparison

ISO/TR 13843:2000 - Water quality - Guidance on validation of microbiological methods

ISO/TS 22117:2010 - Microbiology of food and animal feeding stuffs - Specific requirements and guidance for proficiency testing by interlaboratory comparison

ISO 29201:2012 - Water quality - The variability of test results and the uncertainty of measurement of microbiological enumeration methods

ISO 5725-1:1994 - Accuracy (trueness and precision) of measurement methods and results - Part 1: General principles and definitions

ISO 5725-2:1994 - Accuracy (trueness and precision) of measurement methods and results- Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

ISO 16266:2008 - Water quality - Detection and enumeration of Pseudomonas aeruginosa - Method by membrane filtration

ISO 6461-2:1993 - Water quality - Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) - Part 2: Method by membrane filtration

ISO 6222:1999 - Water quality - Enumeration of culturable micro-organisms - Colony count by inoculation in a nutrient agar culture medium

ISO 7899-2:2000 - Water quality - Detection and enumeration of intestinal enterococci - Part 2: Membrane filtration method

ISO 9308-1:2000 - Water quality - Detection and enumeration of Escherichia coli and coliform bacteria - Part 1: Membrane filtration method

XP T 90-412:2006 - Water quality - Detection and enumeration of pathogenic staphylococci - Membrane filtration method